Naval Submarine Medical Research Laboratory

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MORPHOLOGICAL FEATURES ACCOMPANYING NEURAL DIFFERENTIATION OF NEUROBLASTOMA (N-2A) INDUCED BY MEDIA SERUM AVAILABILITY

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Naval Medical Research and Development Command
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SUMMARY PAGE

THE PROBLEM

To evaluate the efficacy of reduction in media serum availability as a technique for inducing neural differentiation and formation of neuron-like axonal and junctional processes in cultured neuroblastoma cells.

THE FINDINGS

Culture morphological characteristics were monitored for 96 hours after sub-cultivation in support media containing varying concentrations of available serum. Cultivation in media with reduced serum concentrations increased overall proportion of differentiated cells, increased the yield of extended neurite processes, increased the length of the neurites, increased the number of varicosities on the neurites, and increased the number of sites where varicosities on neurites from different cells overlapped or abutted. The results suggest that media serum starvation is an effective technique for inducing growth characteristics in neuroblastoma cell cultures sufficient for neurophysiological study of induced synapogenesis in the culture.

APPLICATION

The results indicate that we can readily grow neuroblastoma cultures which exhibit characteristics usually found only in natural nervous tissue. The induced characteristics include multiple interactions at sites on the neurites occupied by varicosities, as well as overall increases in neuritogenesis. These induced characteristics will enable us to attempt to demonstrate induction of functional synaptic junctions between the cells at the sites of varicosity interaction.

ADMINISTRATIVE INFORMATION

This investigation was conducted under NMRDC Work Unit No. 61153N. MR04101.001-5014, "(U) Cell culture modeling of neurophysiological pathology and brain associative processes." It was submitted for review on 26 January 1989, cleared for publication on 24 July 1989, and designated NSMRL Report No.1140.

ABSTRACT

Previous studies have demonstrated that cultured cloned cells extend neurites after exposure to media supplemented with reduced concentration of serum. The present experiment on cloned neuroblastoma cells (Neuro-2a) corroborates and extends the past studies. N2A cells were cultivated in media containing 10% fetal bovine serum (FBS). After 24 hrs the cells were subcultivated into media containing either 10%, 5%, or 1% FBS; or media containing 10%, 5%, or 1% NU-SERUM V (NSV). The cells were morphologically characterized after 24, 48, 72 and 96 hours incubation. Reduced media serum concentrations increased the proportion of neurally differentiated cells, increased the overall neurite yield, increased the length of the neurites, increased the number of varicosities on the neurites, and increased the number of potentially interactive sites where varicosities on neurites from different cells overlapped or abutted. For all measures, NSV supplementation was found to reliably induce more neuritogenesis than for equal concentrations of FBS. The present experiment suggested that morphological features suitable for long term neurophysiological investigation could be induced by incubation in media containing either 1% NSV or 1% FBS.

INTRODUCTION

Cloned cell lines derived from tumor cells of neural origin, including mouse neuroblastoma (Augusti-Tocco and Sato, 1969; Schubert, Humphreys, Baroni, and Cohn, 1969), rat neuroblastoma (Schubert, Heinemann, Carlisle, Tarikas, Kimes, Patrick, Steinbeck, Culp and Brandt, 1974), and rodent pheochromocytoma (Greene and Tischler, 1976) have been used extensively to model properties of nervous tissue in vitro. Cloned cell lines have been used to study morphological organization (Nelson, 1975), neurotransmitter synthesis and release (Greene and Rein, 1977a,b), and the excitable properties of neurocellular membranes (Dichter, Tischler, and Greene, 1977; Spector, 1981). The focus of each of these studies, however, was modeling a single property of naturally occurring neural tissue (i.e., morphological characteristics, pharmacodynamics, membrane biophysics, etc.). There has been little attempt to model the synaptic properties of organized neural tissue, although simple neuromyal synaptic coupling has been studied using established cultured cell lines (Nelson, Christian and Nirenberg, 1976; Nelson, Neale and McDonald, 1981).

Except for some expected abnormalities given their neoplastic origin, the differentiation pattern exhibited by cloned murine neuroblastoma cells resembles the pattern observed in developing brain neural crest cells (Gurwitz and Cunningham, 1988). Indeed, in pilot studies we have observed the development of varicosities along the neurites of well differentiated mouse Neuro-2A (N2A) neuroblastoma cells as well as a propensity for the varicosities of one neurite to overlap or make physical contact with varicosities along neurites originating from other cells. Exocytotic neurotransmission is a common function served by varicosities in naturally occurring neural tissue, and varicosity arrangements, similar to those we have observed among our N2A cells, have been previously described in cultured pheochromocytoma (PC12) cells (Green and Tischler, 1976). Furthermore, the PC12 cells have been found to be capable of electrical and chemical stimulation coupled neurotransmitter release (Greene and Rein, 1977a,b), the first prerequisite for neurotransmission.

The degree of neuron-like bioelectric activity exhibited by cells from neoplastic lines appears to be directly related to the degree of neural differentiation of the cells (Kuramoto, Werrbach-Perez, Perez-Polo and Haber, 1981; Nelson, 1973). Similarly, there appears to be a strong relationship between the development of neurite varicosities and the extent of neural differentiation in these cells (Greene and Tischler, 1976). We have postulated, therefore, that the established cell line preparation most likely to engage in synaptic coupling and exhibit organizational properties similar to those of normal neural tissue would be one which exhibits the highest degree of neuritogenesis and development of neurite varicosities.

Over the past two decades a number of studies have examined induction of neural differentiation using a variety of methods. Successful methods of inducing nuritogeneses in cells from neoplastic lines include; supplementation

of the incubation media with purified brain glycolipids including bovine gangliosides (Matta, Yorke and Roisen, 1986); stimulation of metabolic activity through addition of dibutyryl c-AMP to the media (Chalayonitis and Greene, 1974); activation by chemical treatment of media with bromodeoxyuridine (Schubert and Jacob, 1970), prostaglandin (Prasad, 1972), or dimethylsulfoxide (Kimhi, Palfrey, Spector, Barak and Littauer, 1976; Prasad, 1975); or inhibition of overall culture growth rate by reduction in media serum concentration (Kaufman and Barret, 1983; Nelson, 1975).

We have chosen to use the latter technique involving serum starvation in the current studies. Prior work in our laboratory corroborates the findings that reliable neuritogenesis, comparable to that induced by chemical media modification, is induced by incubation with low media serum concentrations. Furthermore, recent evidence suggests that the induction of neuritogenesis by chemical modification may occur as a secondary response to a modulation of the activity of serine proteases (Gurwitz and Cunningham, 1988) and that serine protease activity in available serum may control differentiation of neuroblastoma cells in culture (Gibson, Burack, and Picciaro, 1984, Klingman and Hsieh, 1987). Secondly, serum starvation offers an added advantage over chemical modification in that the cell line is maintained under more natural conditions and is less likely to exhibit undesirable changes in structure or function which may be a product of chemical treatment or direct metabolic stimulation.

Additional prior work in our laboratory suggests that neurite expression for mouse neuroblastoma line N2A increases when fetal bovine serum (FBS) is replaced with a more defined serum replacement component Nu-Serum V (NSV) (Messier and Fisher, 1989a,b). Compared to FBS, NSV exhibits significantly lower levels of serine protease activity. Therefore, in addition to development of a maximal culture preparation for neurophysiological investigation, the use of a serum starvation procedure allows us to indirectly examine the role of serine protease inhibition in neuritogenesis by assessing the degree of neural differentiation induced by limiting available concen trations of standard FBS compared to equal concentrations of NSV.

METHODS

Cell Culture. Mouse neuroblastoma cells N2A (American Type Culture Collection, CCL 131) were cultivated in 25 cm^2 plastic flasks (Falcon) in 4 ml Ham's F12 supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5%-CO_2/95%-air. To study the effects of serum deprivation on modification of neurite proliferation, the cells were subcultivated by treatment with trypsin (0.25%) and seeded into 12 25 cm^2 plastic flasks at a density of 2 X 10^5 per flask. The media in 6 flasks consisted of 4 ml of either F12/FBS-10% n=2, F12/FBS-5% n=2, or F12/FBS-1% n=2. The FBS component in an additional 6 flasks was substituted with an equal concentration of NU-SERUM V (NSV) (Collaborative Research, Inc) which is characterized by consistent and well defined hormonal, vitamin, mineral, protein, and nutritional supplementation. Flasks were maintained in an incubator (Hareaus) at 37°C for the duration of the experiments.

Morphology. Each experimental flask was morphologically characterized after 24, 48, 72 and 96 hr intervals from time of subcultivation.

Measurement. At each timed interval, minimum cell diameter (soma) was obtained for 100 cells over at least 10 randomly placed, non-overlapping 300,000 uM^2 sections. Similarly, 25 neurite lengths were obtained for all neurites from the cell observed to possess the longest neurite in each of at least 6 random, non overlapping 300,000 uM^2 sections. All measurements were quantified to a resolution of 1 uM using a morphometric analysis system (CPS-CrystaLogic) interfaced to an Apple II+ computer through a CCTV camera (RCA 1/2" Newvicon-BW) connected to the sideport of an inverted microscope (Nikon, Diaphot).

Attribute Counting. At each timed interval the total number of attached cells, the total number of undifferentiated cells, the total number of cells exhibiting neuritogenesis (i.e., at least one neurite-like arborization at least 10 uM in length), total number of neurites greater than 25 uM, the total number of cells with varicosities (i.e., cells with at least 1 varicosity on at least 1 neurite), and the total number of varicosity crossings (i.e., places where varicosities on one neurite overlapped or abutted varicosities on other neurites) were manually counted for 7 randomly placed, non-overlapping 300,000 uM^2 sections at 20x phase contrast using the inverted microscope. Additionally, the relative percentage of 3 differentiated cell types (i.e., neural-like, epithelial-like and giant) was estimated for 5 randomly, non-overlapping 300,000 uM^2 fields.

Statistical Analysis. All morphological data were analyzed by a statistical software analysis of variance (ANOVA) program with the Duncan post-hoc multiple t-test. The level of significant difference was set at $p < 0.05$.

RESULTS AND DISCUSSION

Differentiation. Three predominant types of attached differentiated cells were observed in the cultures (see Figure 1). These cells were classified as neural-like cells, epithelial-like cells or giant cells based on their shape. Neural-like cells are characterized by uniform somas with one or more well defined neurite projections (Type-1), or by complex somas with distinct arborizations suggesting immature neurite development (Type-2). The neural-like cells were also found to be spatially arranged in formations typical of those seen in developing neural tissue. These spatial formations include well defined separations among somas with any intercellular contact accomplished through neurite extension. Conversely, epithelial-like cells are characterized by smooth flattened oblate-spheroid or teardrop shaped somas with no tendencies toward neurite development. Additionally, the epithelial-like cells were usually observed in clusters of cells arranged in matrices reminiscent of those formed by organ epithelial cells. Giant cells are characterized by their large size (85-200 uM), multiple surface vacuoles, and flattened appearance when contrasted with the other cell types. Unlike the other cell types, the giant cells appear to exhibit no tendency for spatial organization.

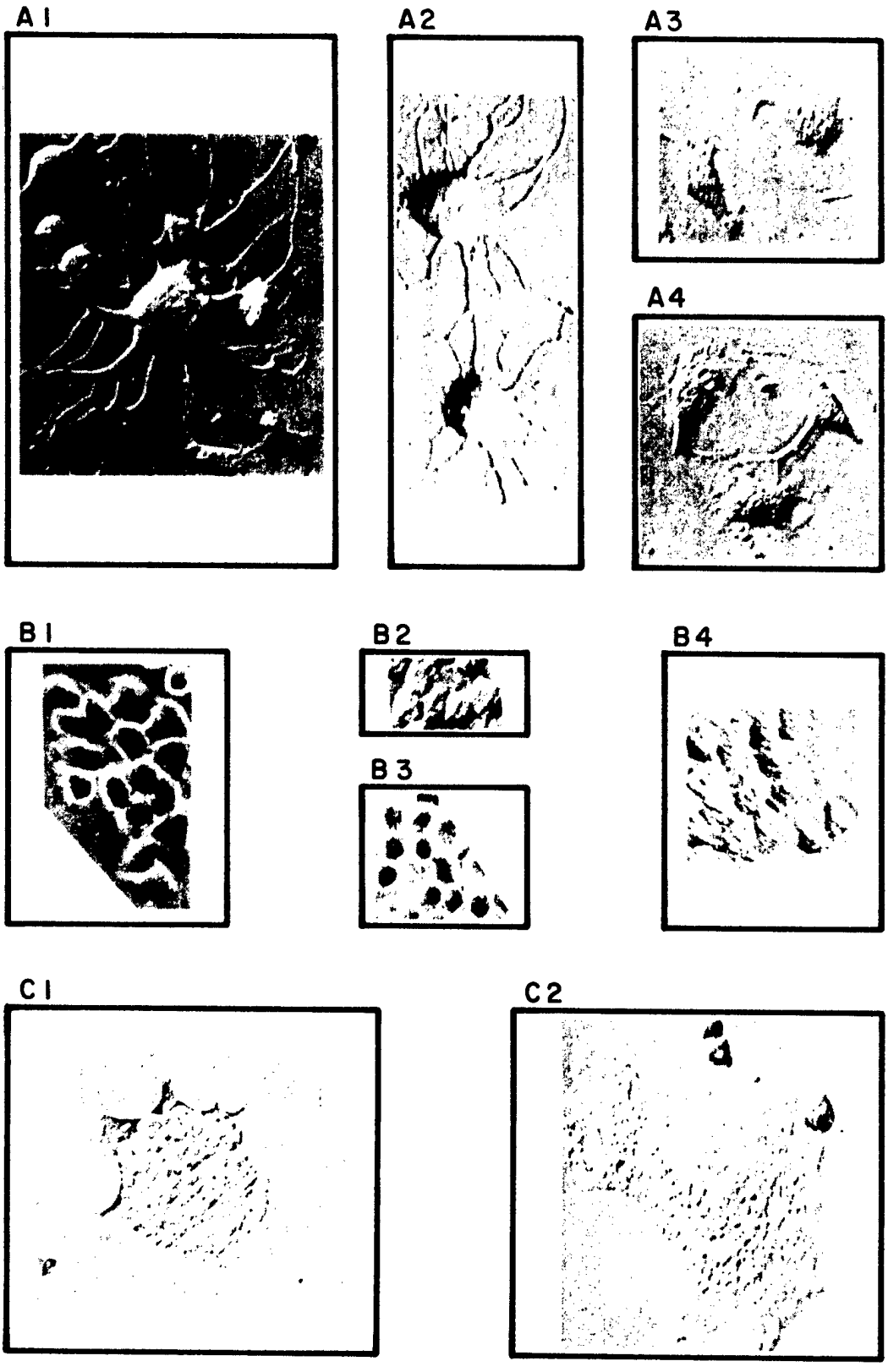

Figure 1

A1, A2 Neural Type 1
A3, A4 Neural Type 2

B1-B4 Epithelial-like
C1, C2 Giant

The approximate distributions of the types of differentiated cells are given in Table 1. Neural-like cells were the most frequently characterized cell type in all cultures except FSB 5% at 48 and 72 hrs, where epithelial cells were most predominant. The proportion of neural-like cells was greatest at the 1% concentrations of FBS and NSV. Concordantly, the greatest proportion of epithelial-like cells were characterized in the 5% concentrations of FBS and NSV. Giant cells were the least frequently characterized cells, seldom accounting for greater than 2% of the differentiated cell population.

The summary of overall differentiation of the cultures is depicted in Figure 2. The percentage of differentiated cells counted was reliably affected by media serum type [$F(1,312)=10.77$, $p<.001$], media serum concentration [$F(2,312)=65.61$, $p<.001$], and time from media subcultivation that the cells were counted [$F(3,312)=31.64$, $p<.001$].

The results of determination of culture neuritogenesis are depicted in Figure 3. The number of neural-like differentiated cells was reliable affected by media serum type [$F(1,312)=7.46$, $p<.01$], media serum concentration [$F(2,312)=103.66$, $p<.01$], and time from media subcultivation [$F(3,312)=18.39$, $p<.001$]. These results are consistent with the conclusion that enhanced neuritogenesis is a consequence of serum deprivation and that NSV media supplementation is superior to FBS media supplementation in induction of neuritogenesis. Furthermore, a reliable interaction between serum type and concentration [$F(2,312)=11.70$, $p<.001$] suggests that media supplementation with NSV promotes a greater degree of differentiation than media supplementation with FBS at lower concentrations of each of the sera.

Number of Neurites. The results are depicted in Figure 4. Both medium serum type [$F(1,312)=11.26$, $p<.001$] and medium serum concentration [$F(2,312)=370.40$, $p<.001$] reliably affected the total number of neurites (>25 uM) counted in each culture. These results support the conclusion that reduction in media serum concentration reliably increases the definition of neuroblastoma culture neuritogenesis and that NSV media supplementation is superior to FBS supplementation in promoting the extension of a greater number of defined neurites. Again, a reliable interaction between serum type and serum concentration [$F(2,312)=4.65$, $p<.01$] suggests that the superiority of NSV media supplementation over FBS in promoting an increase in number of neurites is particularly reliable at lower serum concentrations.

Neurite Lengths. The results are depicted in Figure 5. Both medium serum type [$F(1,576)=10.38$, $p<.001$] and medium serum concentration [$F(2,576)=79.24$, $p<.001$] reliably affected average neurite length. These results suggest that reduction in medium serum concentration reliably increases average neurite length and that NSV media supplementation is superior to FBS supplementation in producing the augmentation. Unlike the other neuritogenesis measures, no reliable interaction was found between serum type and serum concentration [$F(2,576)=0.94$, $p>.10$] suggesting that NSV is generally superior to FBS in augmentation of neurite length.

Number of Cells with Varicosities. The results are depicted in Figure 6. Both media serum type [$F(1,312)=15.69$, $p<.001$] and media serum concentration

TABLE 1

Relative Percentage of Occurrence of Three Differentiated Cell Types (Estimated)

(Time*)	24			48			72			96		
(Type**)	N	E	G	N	E	G	N	E	G	N	E	G
10% FBS	74	24	2	77	22	1	70	28	2	67	32	1
5% FBS	58	41	1	40	58	2	40	59	1	51	49	0
1% FBS	93	5	2	92	7	1	94	6	0	92	8	0
10% NSV	87	12	1	76	24	0	78	20	2	78	21	1
5% NSV	67	31	2	58	41	1	58	42	0	58	41	0
1% NSV	97	1	2	98	2	0	97	2	1	98	2	0

* (Time) : Hours from subcultivation

** (Type) : Classified Cell Type

N = Neural-like Cells (Type-1 + Type-2)
E = Epithelial-like Cells
G = Giant Cells

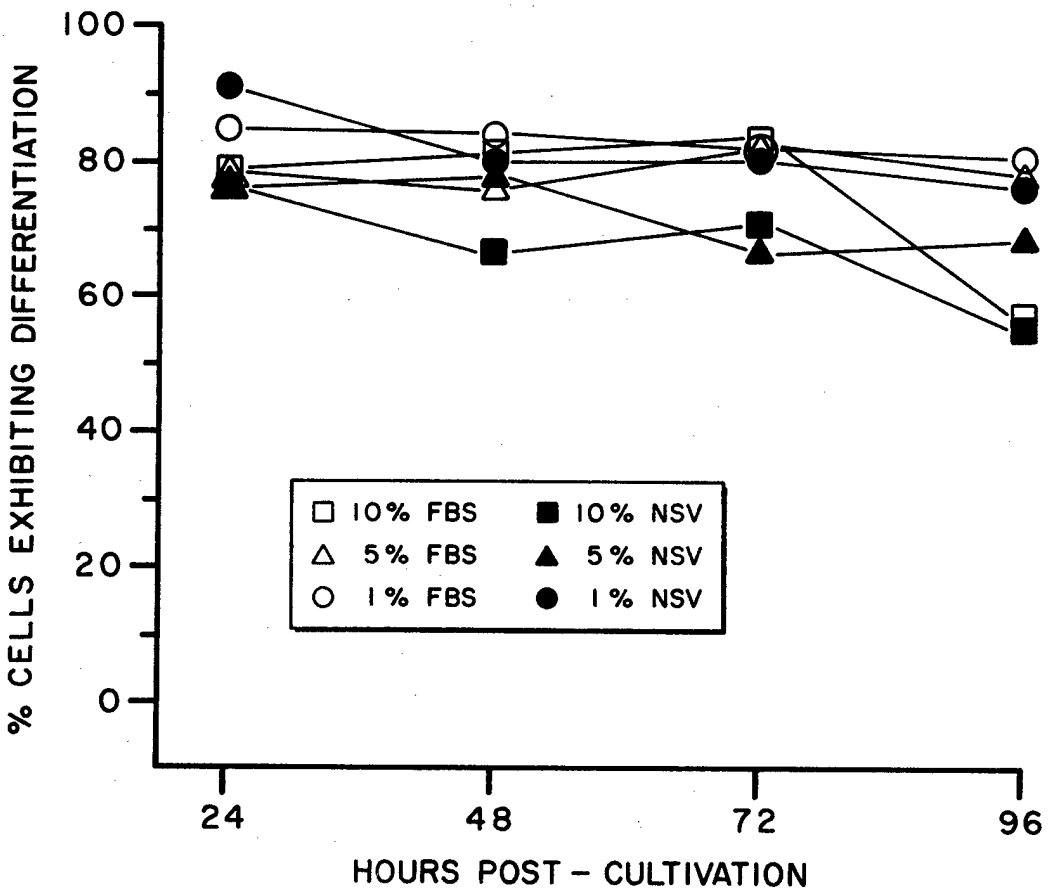

Figure 2. Percentage of cells exhibiting differentiation. Percentages include cells classified as neural type, epithelial type, and giant cells.

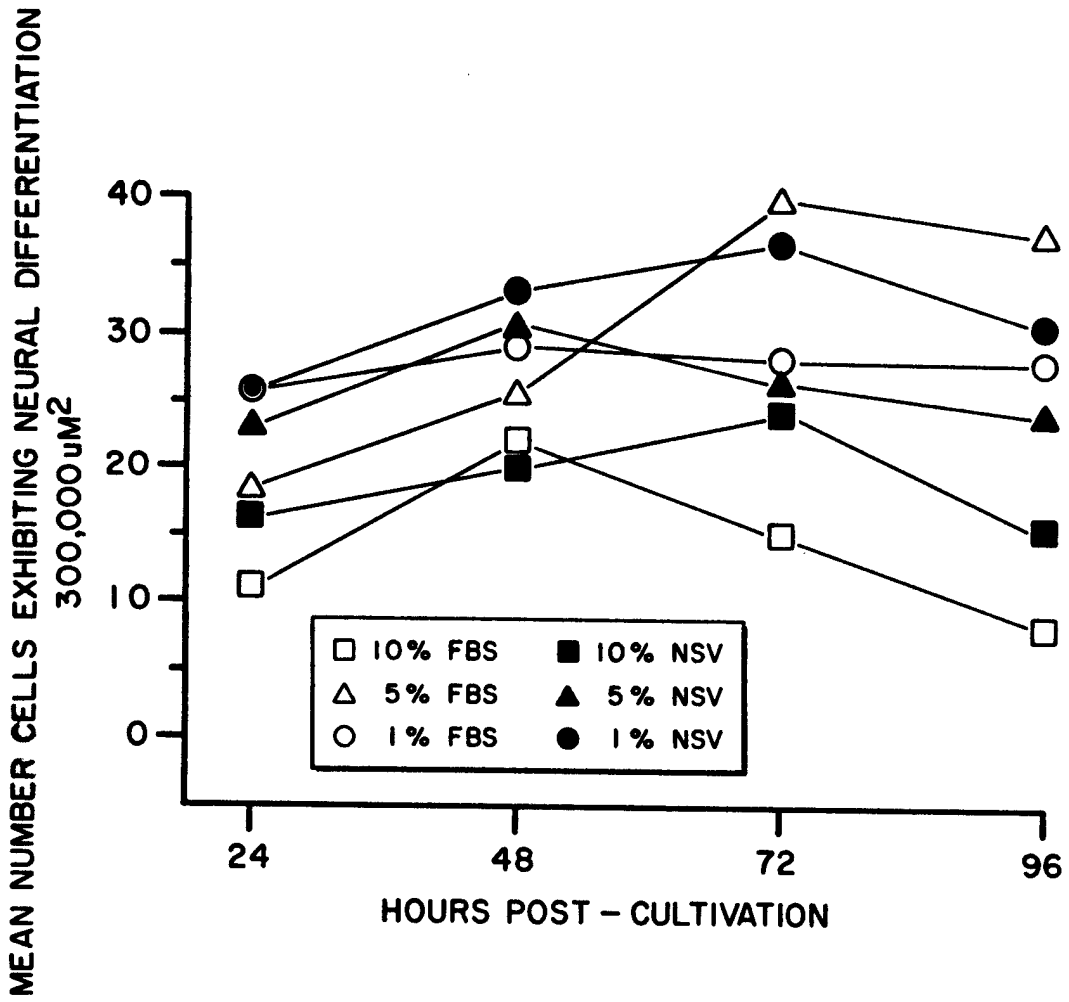

Figure 3. Mean number of cells exhibiting neural differentiation.

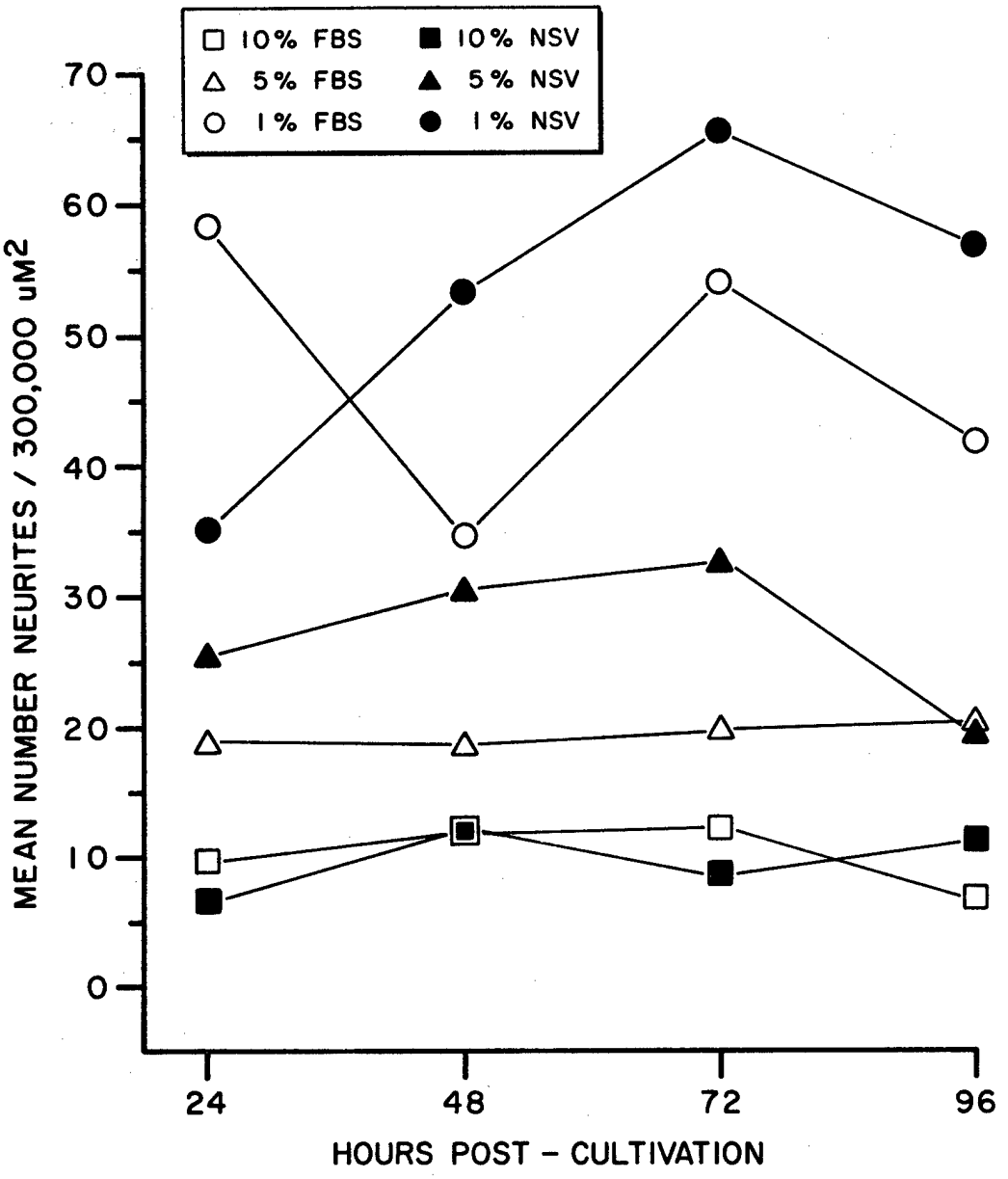

Figure 4. Mean number of neurites counted in 7 non-overlapping 300,000 uM^2 fields.

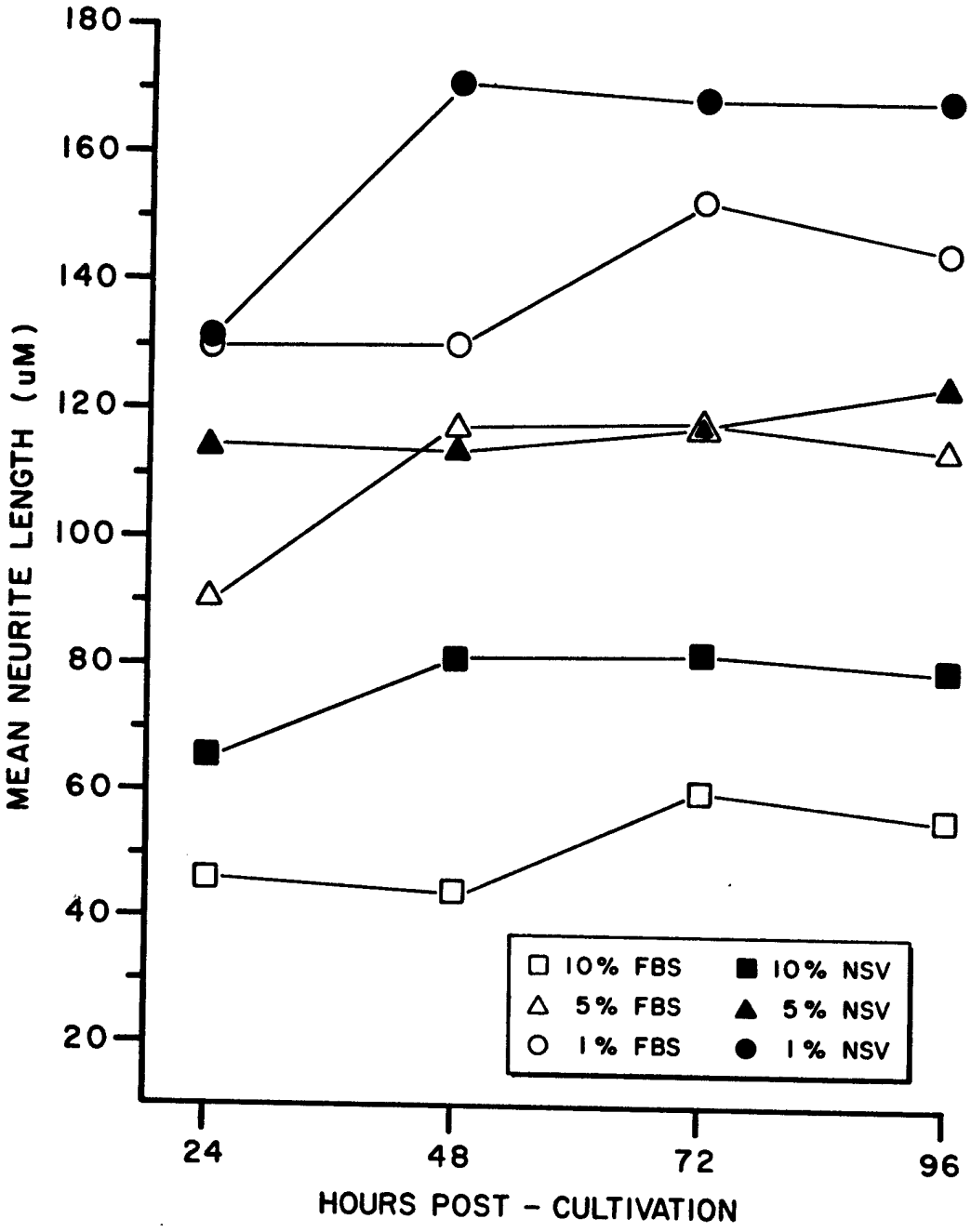

Figure 5. Mean neurite length measured for 150 neurites in at least 6 non-overlapping fields. Neurite length quantized to nearest micron.

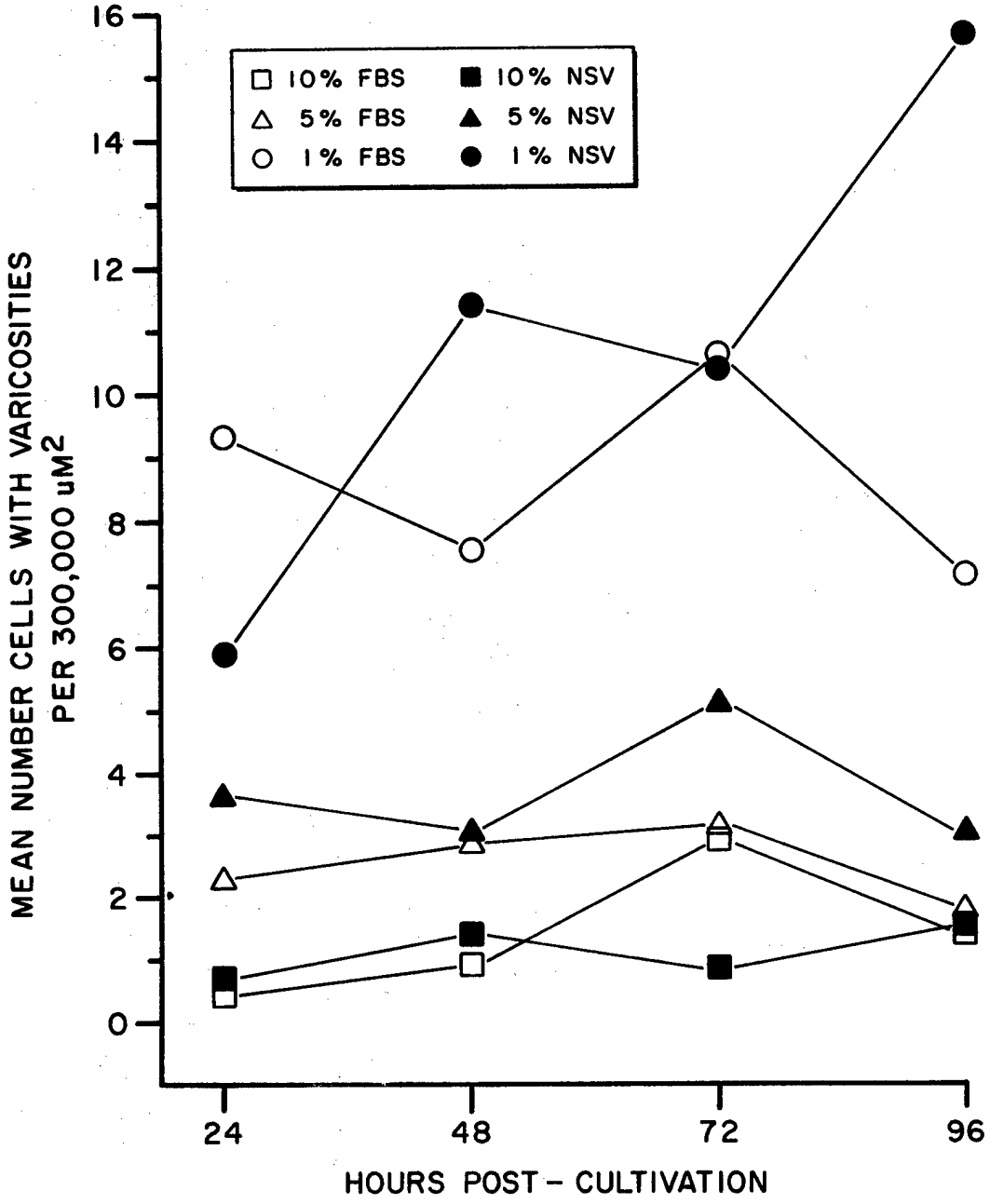

Figure 6. Mean number of cells with varicosities counted over 7 non-overlapping 300,000 uM^2 fields.

[F(2,312)=361.56, p<.001] reliably affected the average number of cells in the culture which exhibited varicosities. Again, the results suggest that reduction in serum concentration promotes varicosity formation along neurites and that NSV is superior to FBS in promoting the effect. A reliable interaction between serum type and serum concentration [F(2,312)=8.36, P<.01] suggests that the superiority of NSV in promoting varicosities might be particularly reliable at low serum concentrations.

Varicosity Crossings. The results are depicted in Figure 7. Only serum concentration was found to reliably affect the number of varicosity crossings in the main analysis [F(2,312)=183.8, p<.001]. Post hoc comparisons of each concentration pair over time, however, revealed a difference between media serum types which was reliable only at the 1% concentrations [F(1,104)=5.67, p<.01], suggesting that at the 1% concentration, NSV was superior to FBS in promoting varicosity crossings.

Soma Diameters. The results are depicted in Figure 8. Both media type [F(1,2376)=9.14, p<.001] and media concentration [F(2,2376)=56.63, p<.001] reliably affected measured cell diameter, whereas there was no reliable effect attributable to time from subcultivation of the cells [F(2,2376)=2.19, p>.05]. A reliable interaction between media type and concentration was also observed [F(2,2376)=9.41, p<.001], and these results are consistent with the overall finding of increased neural differentiation in lower media concentrations as well as with the general finding that NSV promotes a greater degree of differentiation than FBS at lower concentrations. In general, the soma diameters of cells expressing neural differentiation (neural-like) tended to be larger and less varying in diameter than those of undifferentiated cells or of cells expressing other than epithelial-like differentiation.

GENERAL DISCUSSION

The experiment reported here corroborates past findings that reduced media serum concentrations can induce neural differentiation, characterized by neurite outgrowth, in neuroblastoma cell lines. We have also provided evidence which suggests that the neural differentiation induced by serum starvation is not restricted to increasing the proportions of neurally differentiated cells alone. Indeed, the results of this experiment indicate that not only do more cells extend neurites when incubated in low serum media, but that the population of cells extends more neurites, that the neurites are of greater length, that the neurites develop more varicosities, and that the neurites develop more varicosity interactions with neurites from different cells. Furthermore, we have provided evidence which suggests that when conventional fetal bovine serum (FBS) is replaced with the more well defined media supplement NU-SERUM V (NSV) each of these measures of neural differentiation exhibits augmentation.

We are unable to directly compare the results of the current experiment with previous demonstrations of neuritogenesis induced with reduced serum concentration (Gurwitz and Cunningham, 1988; Kaufman and Barret, 1983), due to differences in incubation time, or serum concentrations. The past studies reported the effects of very low serum concentrations (0.0-0.8% FBS) and very short incubation times (<24 hrs). Because the primary goal of our study was

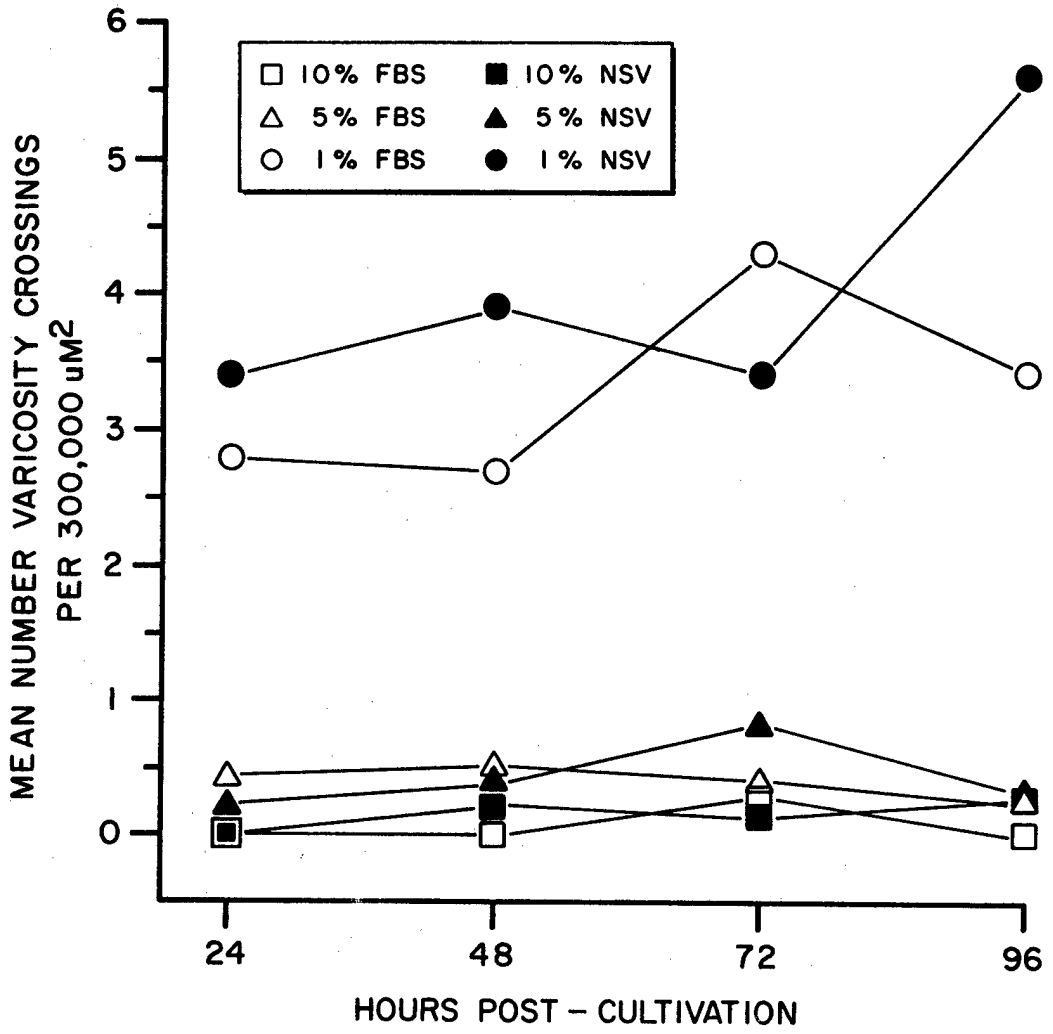

Figure 7. Mean number of varicosity crossings or varicosity abbutments counted over 7 non-overlapping 300,000 uM^2 fields.

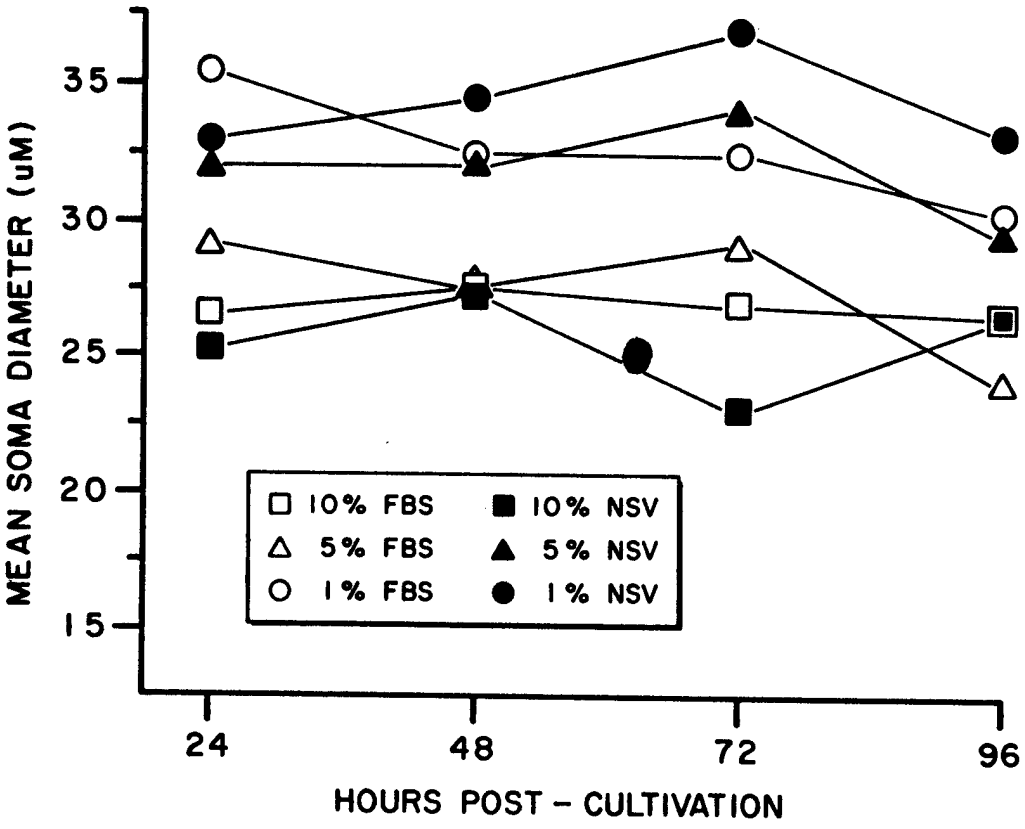

Figure 8. Mean soma diameter measured for 1000 cells averaged over at least 10 non-overlapping 300,000 uM fields. Measurements quantized to nearest micron.

to produce a well differentiated culture suitable for conduct of electrophysiological and neuropharmacological studies which would extend over days, it was necessary to develop a preparation which would exhibit morphological stability lasting for 3 to 4 days. We determined in pilot studies that the minimum NU-SERUM concentration which would allow our cultures the longevity necessary was about 1.0% which restricted our evaluation of lower serum concentrations. We were encouraged, however, by the morphological similarities of our reduced serum preparations and those reported after media chemical modification or metabolic stimulation (Chalayonitis and Greene, 1974; Kimhi et al., 1976; Schubert and Jacob, 1970; Prasad, 1975).

Our current findings also indirectly support the hypothesis that neural differentiation is at least partially under the control of serine protease activity (Gurwitz and Cunningham, 1988; Klingman and Hsieh, 1987). That cells incubated in NSV supplemented media reliably exhibited more neural differentiation than did cells incubated in equal concentrations of FBS, support the possibility that the low protease activity may be responsible for the observed differences. However, it is also possible that the observed morphological differences reflect some other effect produced by the differences in chemical composition of the sera. We are currently investigating this possibility by examining morphological differences induced by FBS and NSV media supplementation in the presence of specific protease inhibitors.

Most past research concerning differentiation of neural cell lines restrict discussion to neuritogenesis. Accordingly, the data analysis in these studies is generally restricted to percentages of cells with neurites of specified lengths (Gurwitz and Cunningham, 1988; Kuramoto et al., 1981; Nelson 1973) or to other neurite related phenomena such as varicosity development (Greene and Tischler, 1976). Our observation of the differentiation of at least 3 different phenotypical groupings of cells warrants consideration. In our 5% FBS 48 and 72 hr cultures, the majority of the cells had differentiated into something other than neural-like cells. High proportions of the cells in the FBS 5% 24 and 96 hr culture groups as well as all four of the 5% NSV measurements reflected similar phenotype expression. The fact that for any concentration of sera, the proportion of the cells scored as non-neural remains nearly constant over the 4 day experimental period suggests that these cells do not further differentiate and exhibit neuritogenesis. Also, the observation that the proportion cells of non-neural phenotype is negligible in the 1% serum concentrations suggests that some property associated with serum concentration might be controlling non-neural phenotypic expression as well as overall neuritogenesis. The observation of 3 distinctive phenotypical groupings with similar organizational characteristics to the ones we observed in our N2A cells have been recently reported in cultured neuroblastoma-ganglion hybrid cells (Dingledine and Boland, 1988). We are currently assessing the possible role of serine protease activity in the determination of neural-phenotypic expression in early cell differentiation.

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Previous studies have demonstrated that cultured cloned cells extend neurites after exposure to media supplemented with reduced concentration of serum. The present experiment on cloned neuroblastoma cells (Neuro-2A) corroborates and extends the past studies. N2A cells were cultivated in media containing 10% fetal bovine serum (FBS). After 24 hrs the cells were subcultivated into media containing either 10%, 5%, or 1% FBS; or media containing 10%, 5%, or 1% NU-SERUM V (NSV). The cells were morphologically characterized after 24, 48, 72 and 96 hours incubation. Reduced media serum concentrations increased the proportion of neurally differentiated cells, increased the overall neurite yield, increased the length of the neurites, increased the number of varicosities on the neurites, and increased the number of potentially interactive sites where

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varicosities on neurites from different cells overlapped or abutted. For all measures, NSV supplementation was found to reliably induce more neuritogenesis than for equal concentrations of FBS. The present experiment suggested that morphological features suitable for long term neurophysiological investigation could be induced by incubation in media containing either 1% NSV or 1% FBS.